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CYTOGENETICS OF THE HAWAIIAN *TELMATOGETON* (DIPTERA)

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ABSTRACT

A cytogenetic analysis of the marine and freshwater species of the Hawaiian Telmatogeton was conducted to determine their evolutionary relationships. Telmatogeton torrenticola, which occupies the islands of Molokai, Maui, and Hawaii, is divided into separate species based on differences in chromosome number, karyotype, and fixed inversions. T. torrenticola-Molokai (n = 4) has three pairs of metacentric chromosomes and a pair of dot chromosomes. T. torrenticola-Hawaii (n = 3/4) has a differentiated sex chromosome system. Males have two pairs of metacentric autosomes, two acrocentric X-chromosomes, and a metacentric Y-chromosome. Females have two pairs of metacentric autosomes and two pairs of acrocentric X-chromosomes. T. torrenticola from East Maui and from West Maui have the same karyotype of six pairs of acrocentric and a pair of dot chromosomes, but they differ from each other by at least six fixed inversions.

In a model of the evolution of the Hawaiian Telmatogeton it is proposed that a marine form was ancestral to the freshwater species. The model also proposes that one of these species may have returned to the sea. Chromosome evolution has involved reduction in chromosome number by centric fusion and the fixation of inversions. T. fluviatilis (n = 7) on Oahu is proposed to be the ancestral freshwater species. It gave rise to T. abnormis (n = 4) and T. hirtus (n = 3/4) on Kauai. T. fluviatilis also gave rise to T. torrenticola-W. Maui, the ancestor of T. torrenticola-Molokai (n = 4), T. torrenticola-E. Maui (n = 7), and T. torrenticola-Hawaii (n = 3/4). It is suggested that the marine species T. pacificus (n = 4) may have returned to the sea with T. torrenticola-Molokai as its ancestor since the two have identical karyotypes.

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INTRODUCTION

In the Hawaiian Islands the genus Telmatogeton is represented by five freshwater and two marine species (Wirth 1947). The freshwater species listed by Wirth are Telmatogeton torrenticola (Terry), found on the islands of Hawaii, Maui, and Molokai, T. williamsi (Wirth) in the Waianae Mountains of Oahu, T. hirtus (Wirth) on Kauai, and T. abnormis (Terry) in the Koolau Mountains of Oahu and on Kauai. Of the two marine species, Wirth states that T. japonicus (Tokunaga) was collected at one site in Hilo Bay on the island of Hawaii and that T. pacificus (Tokunaga) was collected from various sites on Hawaii, Oahu, and Kauai. Additional marine species listed by him are T. simplicipes (Edwards) and T. trochanteratum (Edwards) from southern Chile, T. pusillum (Edwards) from the Marquesas Islands, and T. sancti-pauli (Schiner) and T. minor (Kieffer) from South Africa. In addition, there is the marine species T. macswaini (Wirth) from Mendocino County, California (Cole 1969). Freshwater species of Telmatogeton are found only in the Hawaiian Islands.

All stages of the life cycle of species of the genus Telmatogeton are found in areas of rapidly moving water. The marine forms occupy wave-splashed algae-covered rocks in the intertidal zone. Larvae burrow into the algae and construct tubes from a silklike substance secreted from their salivary glands. The freshwater species are found in waterfalls and rapids of fast-moving mountain streams. They also build tunnels from their salivary gland secretion, generally on bare rocks in the splash zone or in other areas of rapidly moving water. Gut contents from both freshwater and marine species indicate that the larval diet consists of algae. Wirth (1947) feels that the conditions of high aeration, constant moisture, and freedom from waste materials are met for each of the forms by the coast environment and the falls and rapids of mountain streams.

The adults of both forms run about on the rocks seeking mates, copulating, and laying eggs displaying a characteristic zigzag and apparently erratic motion. Although they are poor at flight the adults are able to withstand inundation from splashing water.

Wirth (1947) points out that the genus Telmatogeton appears to be transitional between freshwater and marine environments. Of the members of the subfamily Clunioninae only the genus Telmatogeton has species which are found in fresh water. In the Hawaiian Islands, at least, T. japonicus and T. pacificus are found in regions in which fresh water from mountain streams is mixed with sea water. Wirth (1947) reports that two marine species, T. sancti-pauli and T. japonicus, were found to tolerate life in fresh water in laboratory tests.

In a consideration of the evolution of the Hawaiian Telmatogeton, Wirth (1947) proposes that the marine form T. japonicus gave rise to the freshwater forms. He gives the sequence: T. japonicus → T. abnormis → T. fluviatilis → T. torrenticola → T. hirtus, based on a comparison of tarsal claws in males.

The present study involves an analysis of the polytene, mitotic, and meiotic chromosomes of Telmatogeton larvae for the purpose of:

a) determining the phylogenetic relationships of the Hawaiian Telmatogeton,
b) investigating the transition from marine to fresh water or vice versa,
c) comparing the evolutionary principles displayed by Telmatogeton with those displayed by the Hawaiian Drosophila.

MATERIALS AND METHODS

Collection

Larvae and adults of both marine and freshwater species were collected during the summer of 1973 (Table 1). Collections of T. torrenticola are identified as to island (Molokai, W. Maui, E. Maui, or Hawaii) in this report since there are considerable interisland chromosomal differences within this species. Specific details appear below.

All species of Hawaiian Telmatogeton previously reported from the Islands were collected except T. williamsi and T. japonicus. A search of the Makaha and Waianae Streams in the Waianae Mountains of Oahu on 6 and 13 August 1973 failed to yield T. williamsi. A search made for T. abnormis in the Koolau Mountains was unsuccessful. The marine species T. japonicus was not collected.

Fixation

Larvae were fixed in the field in freshly mixed Carnoy's fixative consisting of 3 parts absolute ethanol and 1 part glacial acetic acid. Best results were achieved by first pulling the head off and then gently squeezing the body before placing the larva into the fixative. Dissection of the larva for salivary glands was generally not required since the glands are extruded into the fixative. Larvae were stored in the fixative in a freezer. Good salivary gland chromosomes were obtained from tissue stored for at least nine months.

TABLE 1. Collections of Telmatogeton, 1973.

Island	Species	Location	Date
Kauai	T. hirtus	Wailua River	13 July
		Makaleha Stream	13 July
		Hanakapiai Stream	11 July
	T. abnormis	Waipoo Falls	11 July
Oahu	T. fluviatilis	Kaluanui Stream	26 June
	T. pacificus	Ala Moana Park	30 June
Molokai	T. torrenticola	Halawa Stream	9 September
West Maui	T. torrenticola	Iao Stream	20 June
		Iao Stream	8 September
		Makamakaole Stream	8 September
		Kahakuloa Stream	8 September
East Maui	T. torrenticola	Hoolawa Stream	8 September
		Kaaiea Stream	8 September
Hawaii	T. torrenticola	Akaka Falls	1 September
	T. pacificus	Hilo Bay	19 June

Stains

The following dilutions of G. T. Gurrs synthetic orcein were used:

Orcein-1 : 2% orcein in 1 part lactic acid and 1 part glacial acetic acid

Orcein-2 : 1 part orcein-1 and 3 parts 45% acetic acid

Orcein-3 : 1 part orcein-1 and 1 part orcein-2

Orcein-4 : 1 part orcein-1 and 3 parts lactic acid and glacial acetic acid (1:1)

Orcein-5 : 2 parts orcein-1, 2 parts orcein-2, and 1 part lactic acid and glacial acetic acid (1:1)

Staining for polytene chromosomes

About 0.5 cm of salivary gland was removed from the larva and stained with or without hydrochloric acid pretreatment. The latter consisted of placing the piece of salivary gland into 1N HCl at room temperature and mechanically removing the lumen contents. The tissue was then treated with 1N HCl at 40°C for 10 minutes, washed in fixative for 10 minutes, stained for 5 minutes in orcein-3, transferred to orcein-4, covered, and squashed lightly.

For preparations without pretreatment, the lumen contents were removed in the fixative, the tissue stained in orcein-5 for 5 minutes, covered, the cover glass tapped gently, and then squashed.

Staining for non-polytene chromosomes

Mitotic material was examined from imaginal disc tissue dissected from the anterior lateral surface of the larvae. This tissue is visible as white spots through the larval cuticle. The tissue was stained in orcein-2 for about 20 minutes, covered, gently heated, and squashed. Meiotic chromosomes were examined from the larval testis stained in orcein-2 for about 30 minutes.

Sexing

Sex was determined after fixation by making a mid-ventral slit in the posterior portion of the larva and examining the gonads. Ovaries are long narrow structures in early larval stages. They grow into massive structures which almost fill the body cavity of the mature larva. The testes are spherical organs which are easily distinguished from the ovary.

Staging

A system was established to determine the relative age of the larvae by examining the size and shape of the imaginal discs which are visible on the sides of the larvae. Four stages in order of increasing age were established.

a) 4-spot - four discs, circular, not touching

b) late 4-spot - four circular discs, touching

c) E-1 - discs have elongated, but not half as wide as segment of posterior set of discs

d) E-2 - discs enlarged to more than half as wide as segment of posterior set of discs.

A fairly good guess as to the sex of the larva could be made by gross examination of the larvae since males are smaller than females. The staging system was useful in finding meiotic material in males. Spermatogenesis occurs very rapidly between stages late 4-spot and E-1. Meiosis has not yet begun in the 4-spot stage and is completed beyond the E-1 stage.

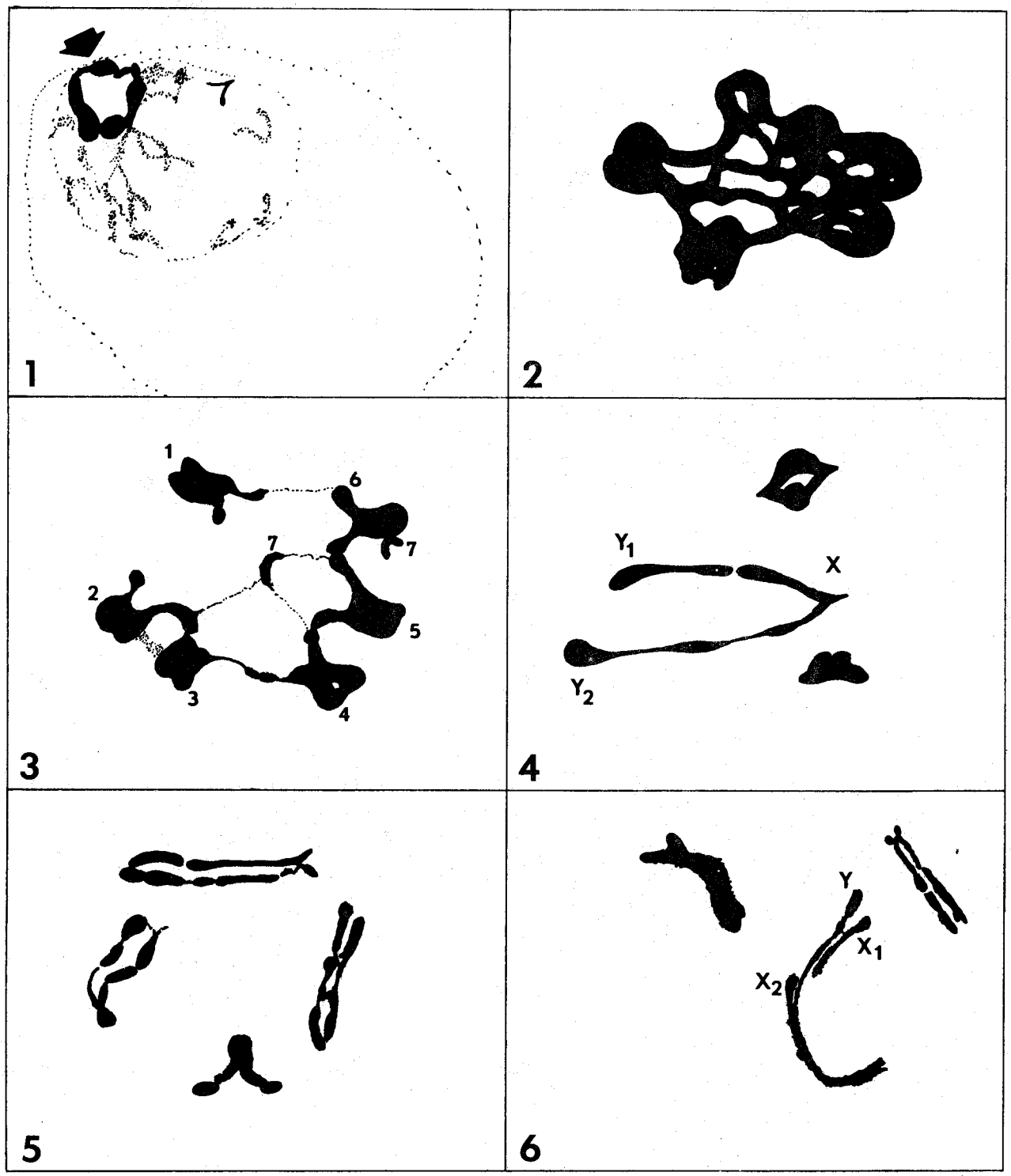
OBSERVATIONS

Non-polytene chromosomes

Mitosis

The following description and interpretation of mitotic prophase is taken from observations of cells from imaginal disc tissue from larvae of T. torrenticola-W. Maui collected from Iao Stream.

The nuclei of interphase cells contain a large circular chromocenter (Fig. 1) which is thought to be composed of the heterochromatin present at the centromeric ends of each of the seven pairs of chromosomes. During early prophase the chromosomes are attached to and radiate out from the chromocenter. The homologous chromosomes are tightly paired. As the euchromatic material of the chromosomes condenses and becomes darker, the chromocenter loses its circular shape. By mid-prophase the chromocenter has disappeared but the chromosomes still radiate out from a central site giving a spokelike appearance. In the hub area each chromosome has a short dark-staining region. As the chromosomes continue to shorten the mitotic figures take on a complicated lacelike configuration (Fig. 2) in which the chromosomes now exhibit no differentiation in staining intensity. At metaphase the chromosomes are still paired and touch one another, forming a circle (Fig. 3). Dot chromosomes are generally located in the center of the circle. Anaphase cells are



FIGS. 1-6. Drawings of the non-polytene chromosomes of the Hawaiian Telmatogeton (from photos). FIG. 1. Interphase cell with large chromocenter, T. torrenticola-W. Maui (arrow); FIG. 2. Mitotic prophase showing lacelike pattern, T. torrenticola-W. Maui; FIG. 3. Mitotic metaphase, T. fluviatilis; FIG. 4. Meiotic metaphase-I in T. hirtus male showing the XY_1Y_2 trivalent; FIG. 5. Meiotic diplotene in T. abnormis male; FIG. 6. Mitotic prophase showing X_1X_2Y trivalent in T. torrenticola-Hawaii.

infrequent and are generally poorly spread.

It is difficult to count the chromosomes in prophase and metaphase since they are grouped together in the spoke, lace, or circle configurations, but occasionally cells with well-spread chromosomes are found. Chromosomes are easier to count in species with a lower chromosome number. The location of the centromere is also difficult to determine since anaphase chromosomes are poorly spread. The use of colcemid and sodium citrate may be of value in solving these problems in future studies.

Meiosis

Observations of male meiosis were of great value in confirming the chromosome number in T. torrenticola-W. Maui, T. hirtus, T. abnormis, and T. pacificus. Testes with cells in meiotic stages beyond pachytene are found only in larvae which are between the late 4-spot and E-1 stages of larval development. Earlier stages contain testes with only zygotene and pachytene cells and the testes of later stages contain only sperm cells. Large samples of larvae are thus required to insure the availability of meiotic material.

The various stages of meiosis are not very different from those found in other species. The chromosomes of diplotene cells of T. hirtus become very large and diffuse giving the appearance of lampbrush chromosomes. Pachytene cells from all species examined exhibit chromosome arms extending out from a large dense chromocenter similar to the situation found in mitotic prophase. Diagrams of diplotene and metaphase-I cells appear in Figs. 4 and 5.

Chromosome number

The Telmatogeton species examined fall into four groups relative to chromosome number and karyotype. Since centromeres and arm lengths are difficult to determine, those chromosomes with interstitial centromeres are referred to as metacentric chromosomes and those with centromeres very close to the end of the chromosome are referred to as acrocentric chromosomes. In future studies the metacentric chromosome category may be broken down into metacentric and submetacentric chromosomes.

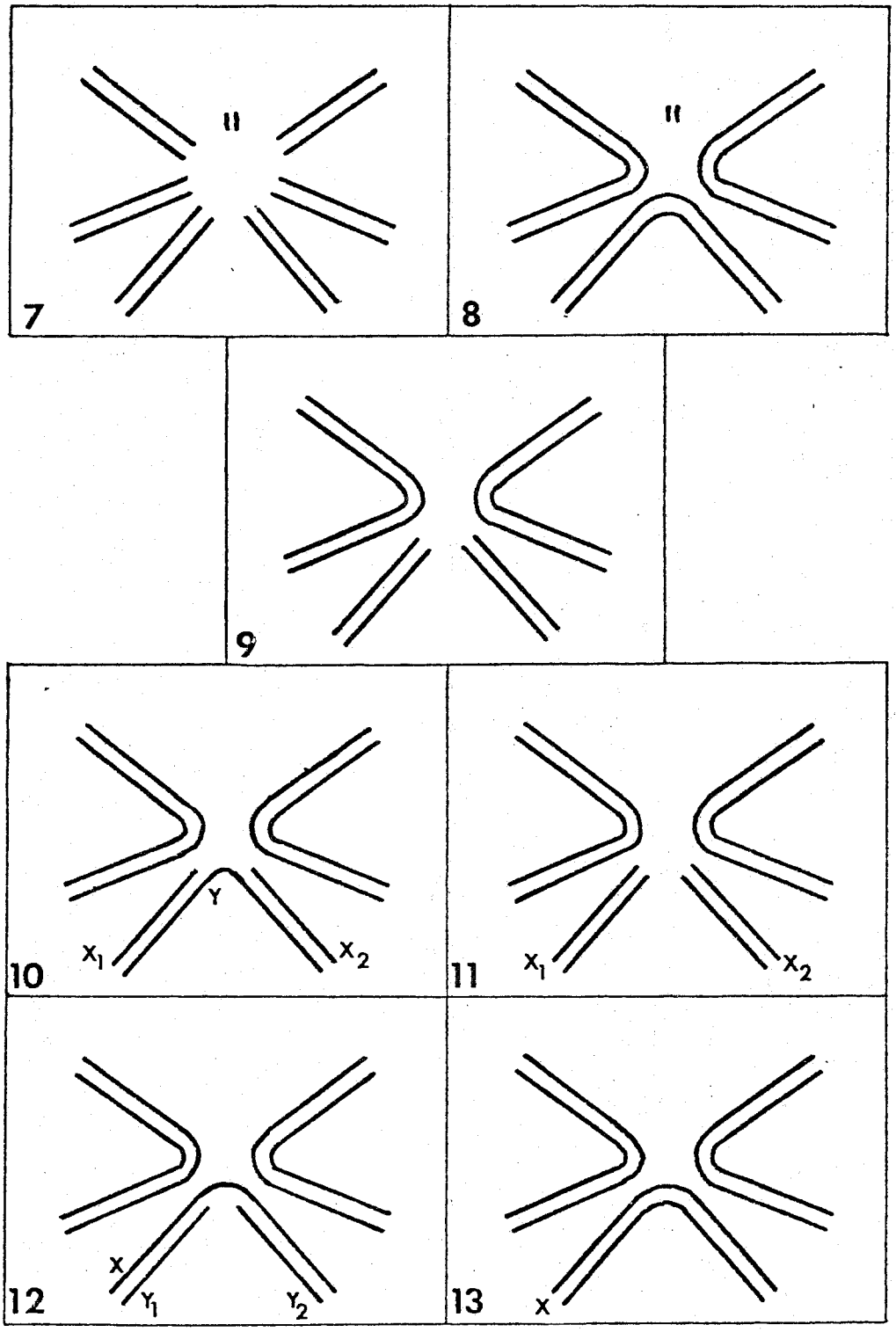
The chromosome groups are:

a) 6 pairs of acrocentric and 1 pair of dot chromosomes (n = 7; Fig. 7)

T. fluviatilis

T. torrenticola-E. Maui

T. torrenticola-W. Maui



FIGS. 7-13. Approximate karyotypes of Telmatogeton species.
FIG. 7. T. fluviatilis, T. torrenticola-East Maui, and T. torrenticola-West Maui (2n = 14); FIG. 8. T. torrenticola-Molokai and T. pacificus (2n = 8); FIG. 9. T. abnormis (2n = 8); FIG. 10. T. torrenticola-Hawaii male (2n = 7); FIG. 11. T. torrenticola-Hawaii female (2n = 8); FIG. 12. T. hirtus male (2n = 7); FIG. 13. T. hirtus female (2n = 6).

b) 3 pairs of metacentric and 1 pair of dot chromosomes (n = 4; Fig. 8)

T. torrenticola-Molokai

T. pacificus

c) 2 pairs of metacentric and 2 pairs of acrocentric chromosomes (n = 4; Fig. 9)

T. abnormis

d) species with sex chromosomes; males with 5 metacentric and 2 acrocentric chromosomes, females with either 3 pairs of metacentric chromosomes or 2 pairs of metacentric and 2 pairs of acrocentric chromosomes

T. torrenticola-Hawaii (n = 3/4; Fig. 10 & 11)

T. hirtus (n = 3/4; Fig. 12 & 13)

Polytene chromosomes

Quality

The quality of the polytene chromosomes was generally excellent in T. torrenticola and T. hirtus but only fair in T. abnormis and T. fluviatilis. T. pacificus has polytene chromosomes which are thin, have poorly defined bands, and are so far impossible to analyze. In general, the larger animals give the better cytological preparations. In T. fluviatilis, for example, only animals over 10 cm in length are suitable for cytological examination. Smaller larvae have poorly developed polytene chromosomes.

The elements

Six obvious polytene chromosome elements may be observed in all of the species. The elements have been assigned letters based on the apparent T. hirtus karyotype of two pairs of metacentric and two pairs of acrocentric chromosomes. The arms of the metacentrics are labeled elements A and B, C and D. The letters EF and GH are used for the acrocentrics. For each of these the second letter is used to stand for the yet unobserved short arm of the acrocentric chromosomes. The six elements are thus designated A, B, C, D, EF, and GH. In all of the species examined except for T. hirtus there is an additional mass of chromatin not organized into a normal looking polytene chromosome. This mass of diffuse, amorphic chromatin may represent an additional and not so obvious chromosome element.

Heterochromatin

A mass of heterochromatin which is variable in its appearance from species to species and between animals of the same species may be found at one end of each element and in an interstitial region of element A (Table 2). In some cells of T. fluviatilis and T. abnormis all of the elements radiate from a small chromocenter

TABLE 2. Comparative polytene chromosome features.

Species	Segment between centric and interstitial heterochromatin, element A	Element A interstitial heterochromatin	Centric heterochromatin
T. hirtus	attached	dense	dense in elements A-B & C-D, variable in EF and GH
T. abnormis	attached	dense	dense, variable
T. fluviatilis	absent	absent	dense, variable
T. torrenticola-Molokai	attached	dense	light, variable
T. torrenticola-W. Maui	separate or loosely attached	light, variable	light, variable
T. torrenticola-E. Maui	situation not clear	situation not clear	light to absent
T. torrenticola-Hawaii	separate or loosely attached	dense, deep notch	light, variable

formed from the terminal heterochromatin of each element. This heterochromatin is thus believed to contain the centromere and is referred to as the centric heterochromatin. In T. hirtus there is a zone of light-staining chromatin between the centric heterochromatin of elements A and B in one of the metacentric chromosomes. The caps of centric heterochromatin of elements C and D touch one another without the clear area as in elements A and B. There is no obvious centric heterochromatin in element EF and there is a large mass of heterochromatin at one end of the GH element.

Element A of all species except T. fluviatilis contains a mass of interstitial heterochromatin which varies in appearance from a large, dense mass of material to almost complete absence. It may appear as two bands or as a single dense band which is frequently notched. In the collection of T. torrenticola-W. Maui from Iao Stream the interstitial heterochromatin is very diffuse and there is a tendency for the two sections of chromosome on either side of the heterochromatin to be separated.

Puffs, constrictions, and nucleolar attachments

Two puffs are found in all of the freshwater species. One is a small puff in the B element, the other a large puff in the D element. There is also a very large fan-shaped puff at one end of the EF element of T. hirtus. This may represent the location of the mass of amorphic chromatin found in all other freshwater species.

In T. torrenticola-W. Maui there is a deep constriction close to the centromeric end of the B element. In some cells the constriction cuts the B element into two pieces giving the appearance of an additional short element.

In T. hirtus the nucleolus is attached to the centric heterochromatin of element GH. In the other freshwater species the nucleolus is generally free from any element association. Some cells of T. torrenticola show an association of the nucleolus with the interstitial heterochromatin of the A element.

Association of elements into chromosomes

The association of polytene elements into chromosomes is the same in T. hirtus and T. abnormis. In T. hirtus the centric heterochromatin of elements A and B are tightly joined together by a region of light-staining chromatin. Elements C and D are loosely attached at their respective centric heterochromatic bands without any space in between. In female larvae, elements EF and GH are loosely attached in the region of centric heterochromatin and the large puff. The EF and GH association in males is complex and is discussed below under sex chromosomes.

In T. abnormis there is also an association of elements A and B but there is

no light-staining area between the centric heterochromatic bands as in T. hirtus. The polytene elements of T. abnormis meet in a poorly defined chromocenter. In the squash preparation the appearance of chromosome spreads is variable; all of the elements may be attached by their centric heterochromatin into a chromocenter or the elements may be scattered with a general association of elements A and B, C and D. There is no attachment between elements EF and GH.

The association of polytene elements into chromosomes in T. torrenticola-Molokai (n = 4) and T. torrenticola-Hawaii (n = 3/4) is not known. Collections from both sites have polytene elements which have poorly defined centric heterochromatin and elements which are loosely joined at the chromocenter. In cells in which the elements are broken away from the chromocenter the elements do not show any consistent association. The mitotic/meiotic chromosomes indicate that the elements should be joined together into chromosomes. So, for example, it is not known if element A forms a chromosome with element B, C, D, EF, or GH. The problem of element association in these two species may be due to the diffuse nature of the centric heterochromatin which allows the arms of the chromosomes to fall apart.

Species with well-defined centric heterochromatin (T. hirtus and T. abnormis) tend to have well-defined element associations. Species with poorly developed centric heterochromatin do not reveal their polytene element associations.

Analysis of banding patterns

The most frequent banding pattern for each element was chosen as the "standard" sequence. The standard is indicated by the symbol "+" and inversion changes from the standard are indicated by the element letter and a subscript number. The approximate locations of the inversions relative to standard are given in Fig. 14. The list of fixed and polymorphic inversions found in each species is given in Table 3. A phylogenetic relationship of species as determined from fixed inversions is shown in Fig. 15. Elements C, D, EF, and GH in T. hirtus and elements C and D in T. torrenticola-E. Maui are so complex that the analysis of inversion changes to standard is not yet possible.

T. torrenticola-Hawaii and T. torrenticola-E. Maui are the only species which share an inversion (GH_4). Each of the other species has its own unique set of fixed and polymorphic inversions. T. torrenticola-W. Maui contains all of the standard chromosomes. The other species have fixed inversions relative to the standard. T. fluviatilis is closest to the standard with one fixed inversion; T. abnormis and T. torrenticola-Molokai each have three, T. torrenticola-Hawaii has two and

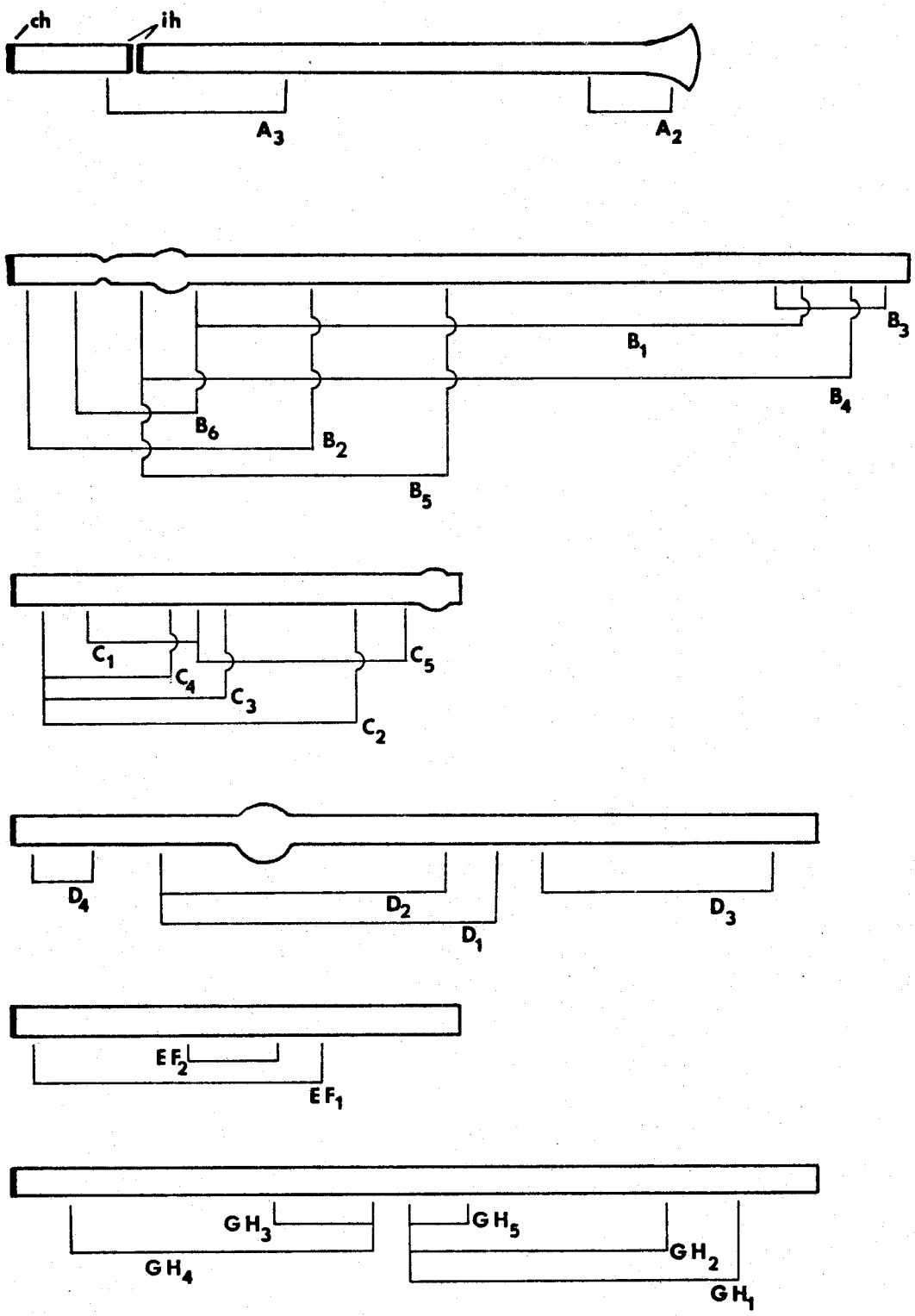


FIG. 14. Diagram of the approximate positions of inversions in the polytene elements relative to the T. torrenticola-W. Maui standard. Bars represent standard arrangement and brackets indicate approximate limits of inversions. Centromeric heterochromatin appears to the left. ch = centric heterochromatin ih = interstitial heterochromatin

TABLE 3. Comparison of polytene chromosome band sequences between species. Single symbol (+ = standard; letter = inversion) indicates fixed condition, letter/+ indicates inversion is polymorphic.

Species	Polytene Element					
	A	B	C	D	EF	GH
T. hirtus	A_1/+	+	Complex	Complex	Complex	Complex
T. abnormis	+	B_2	C_3 C_4	+	+	+
T. fluviatilis	+	+	C_5	+	+	+
T. torrenticola-Molokai	A_2	+	C_1/+ C_2/+	D_1/+ D_4	EF_1	+
T. torrenticola-W. Maui	+	B_1/B_3/+	+	D_2/+	+	GH_1/+ GH_5/+
T. torrenticola-E. Maui	A_3	B_5 B_4/+	Complex	Complex	EF_2	GH_2 GH_3 GH_4
T. torrenticola-Hawaii	+	B_6/+	+	D_3	+	GH_4

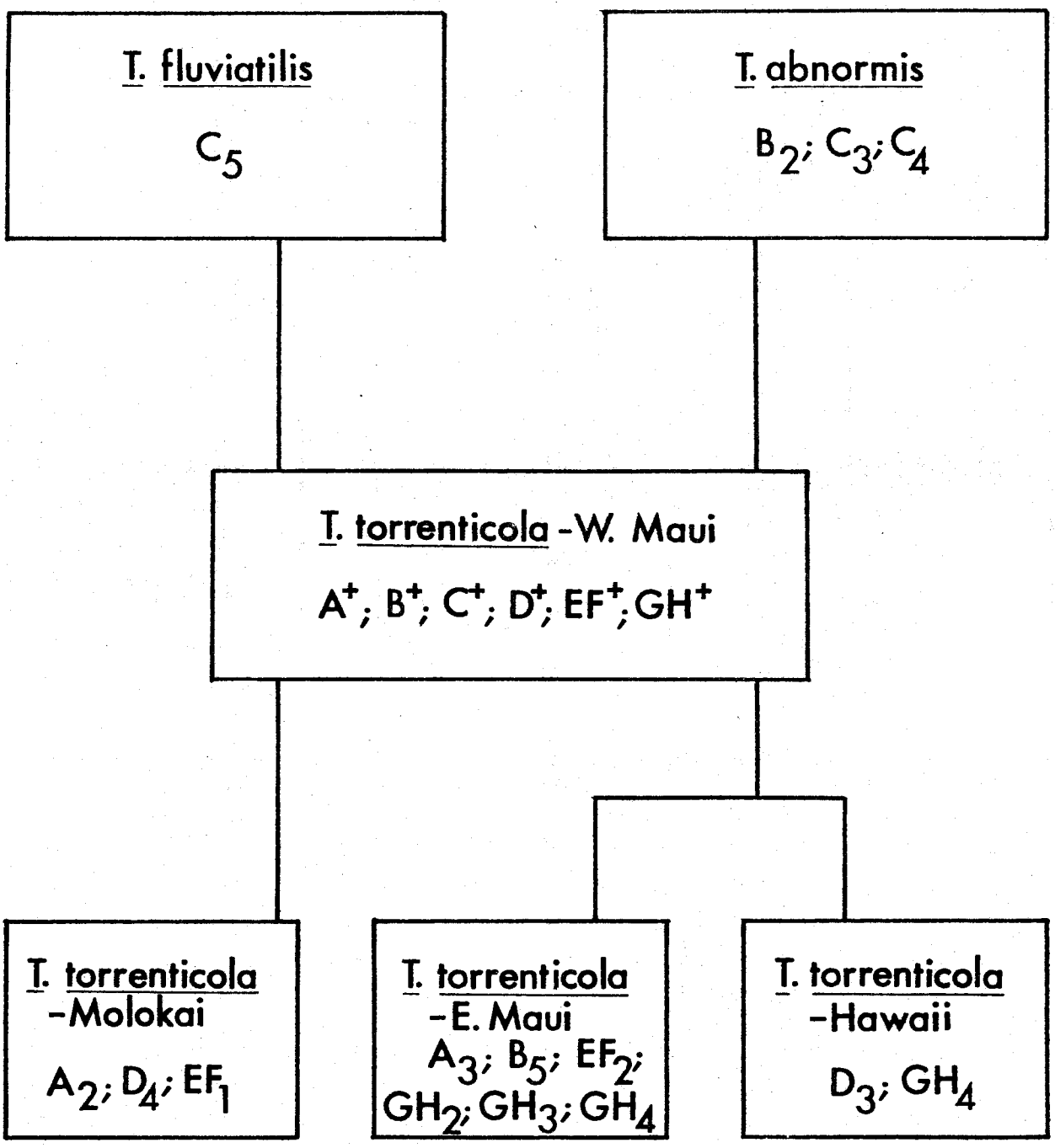


Fig. 15. Relationships between species as determined by fixed inversions. Standard sequence for each element is indicated by a letter and a superscript "+"; fixed inversions relative to the standard are indicated by the element letter and a subscript number.

T. torrenticola-E. Maui has at least six.

Inversion polymorphism

Inversion polymorphism was observed in all species except for T. fluviatilis (10 animals examined) and T. abnormis (28 animals examined). Inversion frequency data fit closely to those expected according to the Hardy-Weinberg equilibrium (Table 4).

T. torrenticola-W. Maui is polymorphic for inversions B_1, B_3, D_2, GH_1, and GH_5. T. torrenticola-W. Maui from Iao Stream (18 animals) is polymorphic for B_1 and B_3 inversions and fixed inverted for D_2 and GH_1, and fixed standard for GH_5. T. torrenticola-W. Maui from Makamakaole Stream (16 animals) is polymorphic for B_3, D_2, and GH_1 inversions and is fixed standard for B_1 and GH_5. The only specimen from the Kahakuloa Stream collection is homozygous standard for B_1, homozygous inverted for D_2 and B_3, and heterozygous for GH_1 and GH_5. One animal from the Makamakaole Stream collection is standard for all inversion sequences. Frequency data are available for all of these inversions except for B_3 in the Iao Stream collection (Table 4).

There are two cases of striking differences in inversion frequency between streams on the same island. In T. torrenticola-E. Maui the frequency of inversion B_4 is 0.750 in Hoolawa Stream (14 animals) and 0.955 in Kaaiea Stream (22 animals). In T. hirtus inversion A_1 has a frequency of 0.033 in Wailua River (30 animals) and 0.283 in Hanakapiai Stream (46 animals). There are not enough data to determine if there is an inversion frequency cline on these islands.

Sex chromosomes

Differentiated sex chromosomes were found in the salivary gland, imaginal disc, and testis cells of T. hirtus and in the salivary gland but not in the testis or imaginal disc cells of T. abnormis. There is some evidence for the presence of sex chromosomes in the imaginal disc cells of T. torrenticola-Hawaii. The other Telmatogeton species lack differentiated sex chromosomes.

T. hirtus has an XY_1Y_2 sex chromosome system. Mitotic cells from females contain two pairs of metacentric autosomes and one pair of metacentric X-chromosomes (n = 3). Prophase-I cells from males contain two metacentric autosomal bivalents and a sex chromosome trivalent composed of one metacentric X-chromosome and two acrocentric Y-chromosomes (Fig. 4). During anaphase-I the trivalent disjoins, with the X-chromosome moving to one pole and the two Y-chromosomes moving to the opposite

TABLE 4. Inversion polymorphism in Telmatogeton species. Site codes: IAO = Iao Stream, MAK = Makamakaole Stream, HOO = Hoolawa Stream, KAA = Kaaiea Stream, HAL = Halawa Stream, WAI = Wailua River, HAN = Hanakapiai Stream. Expected values according to Hardy-Weinberg equilibrium.

Species	Site	Inversion	No. Animals	Number/ Observed/ Expected	Chromosomes I = inversion + = standard: Inversion frequency I	+	Genotype I/I	I/+	+/+
T. torrenticola-W. Maui	IAO	B_1	18	No.			0	5	13
				Obs.	.14	.86	.000	.278	.722
				Exp.			.020	.241	.740
	MAK	B_3	16	No.			3	8	5
				Obs.	.438	.563	.188	.500	.313
				Exp.			.192	.493	.316
		D_2	16	No.			6	8	2
				Obs.	.625	.375	.375	.500	.125
				Exp.			.391	.469	.141
		GH_1	16	No.			5	7	4
				Obs.	.531	.469	.313	.438	.250
				Exp.			.282	.498	.220
T. torrenticola-E. Maui	HOO	B_4	14	No.			8	5	1
				Obs.	.750	.250	.571	.357	.071
				Exp.			.563	.375	.063
	KAA	B_4	22	No.			20	2	0
				Obs.	.955	.045	.909	.091	.000
				Exp.			.912	.086	.002
T. torrenticola-Molokai	HAL	C_4	21	No.			14	5	2
				Obs.	.786	.214	.667	.238	.095
				Exp.			.618	.336	.046
		C_5	21	No.			17	3	1
				Obs.	.881	.119	.810	.143	.048
				Exp.			.776	.210	.014
		D_1	20	No.			18	2	0
				Obs.	.950	.050	.900	.100	.000
				Exp.			.903	.095	.003
T. hirtus	WAI	A_1	30	No.			0	2	28
				Obs.	.033	.967	.000	.067	.933
				Exp.			.001	.064	.935
	HAN	A_1	46	No.			4	18	24
				Obs.	.283	.717	.087	.391	.522
				Exp.			.080	.406	.514

pole. Disjunction of the bivalents is normal. Male meiosis yields sperm containing two autosomes and an X-chromosome (n = 3) and sperm containing two autosomes and two Y-chromosomes (n = 4). The karyotypes for T. hirtus are illustrated in Figs. 12 and 13.

The polytene chromosomes of T. hirtus males are identical to those of the female except for the EF element. In males the EF homologs are identical and tightly paired for about one-quarter of their length distal to the centromere. The remaining segment of the element proximal to the centromere consists of unpaired and nonidentical homologs. One homolog segment is identical to that portion of the EF element of females and the other homolog segment is composed of a mass of densely stained chromatin largely devoid of bands. In some cells there is a strand of chromatin joining the nonheterochromatinized proximal section of the EF element and the proximal portion of the GH element. It is proposed that the X-chromosome is composed of fused EF and GH elements. One Y-chromosome is a partially heterochromatinized acrocentric EF element and the other Y-chromosome is an acrocentric GH element.

The sex chromosomes of T. abnormis are not as striking as those of T. hirtus in that neither the meiotic nor the mitotic chromosomes of T. abnormis reveal any obvious differences between the sexes (Figs. 5 & 9). The polytene elements are identical between the sexes except in males there is an unpaired region in the EF element adjacent to the centromere. The unpaired segment of one homolog is identical to the banding sequence of the female and represents the X-chromosome. The unpaired segment which represents the Y-chromosome is slightly longer than the X-chromosome segment and has a unique banding sequence.

The analysis of several mitotic cells from T. torrenticola-Hawaii was inconclusive relative to determining the sex chromosomes (Fig. 6). There is, however, an indication that males may have two pairs of autosomes and a sex chromosome trivalent composed of two X-chromosomes and one Y-chromosome (X_1X_2Y). If this is the case males will produce sperm with two autosomes and two X-chromosomes (n = 4) and sperm with two autosomes and a Y-chromosome (n = 3). Female mitotic cells appear to have two pairs of autosomes and two pairs of X-chromosomes (X_1X_1, X_2X_2). Females should produce eggs with two autosomes and two nonhomologous X-chromosomes (n = 4). Tentative karyotypes for T. torrenticola-Hawaii are illustrated in Figs. 10 and 11. Meiotic material of suitable stages for verification of this system were not found in the collection.

DISCUSSION AND CONCLUSIONS

The following items of information, isolated from the main body of data available from this preliminary study, are most important in the construction of a tentative evolutionary scheme for the Hawaiian Telmatogeton.

1. The inversion relationships of the species examined (Fig. 15) show that T. torrenticola-W. Maui has a central position with T. fluviatilis, T. abnormis, and T. torrenticola-Molokai extending out separately and with T. torrenticola-E. Maui and T. torrenticola-Hawaii extending out as a branched line.
2. Both T. abnormis and T. hirtus have the association of elements A and B, C and D into metacentric chromosomes. The element associations of T. torrenticola-Molokai and T. torrenticola-Hawaii are not known.
3. The species fall into four groups relative to chromosome number.
   a. T. fluviatilis, T. torrenticola-W. Maui, and T. torrenticola-E. Maui have seven pairs of chromosomes including a pair of dot chromosomes.
   b. T. torrenticola-Molokai and T. pacificus have four pairs of chromosomes including a pair of dot chromosomes.
   c. T. abnormis has four pairs of chromosomes, no dot chromosome.
   d. T. hirtus and possibly T. torrenticola-Hawaii have a 3/4 chromosome system based on differentiated sex chromosomes. They do not have the dot chromosome.
4. The sex chromosome system involves a simple change in element EF in T. abnormis and a complex change in elements EF and GH in T. hirtus. The sex chromosome system of T. torrenticola-Hawaii is not similar to that of T. hirtus.

Changes in chromosome number have obviously occurred frequently in the evolution of the Hawaiian Telmatogeton. To understand the phylogeny of the species, it is extremely important to know whether there have been systematic decreases in number, increases in number, or both. Thus, there may have been fusions of acrocentric chromosomes into metacentric chromosomes to reduce the chromosome number, dissociations of metacentric chromosomes into acrocentric chromosomes to increase the chromosome number, or both fusions and dissociations. Examples of these types of chromosome change in animal evolution are discussed in White (1973).

The ideal chromosomes for centric fusion are acrocentric chromosomes made up of a long euchromatic arm and a short heterochromatic arm. This is exactly the situation

for the acrocentric chromosomes in Telmatogeton species. A reciprocal translocation between two nonhomologous chromosomes with break-points within the centric heterochromatin may produce a large euchromatic metacentric chromosome and a small heterochromatic chromosome which may be lost from the cell line. Fixation of the large metacentric chromosome in a population would decrease the haploid chromosome number by one. Numerous examples of this type of chromosome change are found in the literature (White 1973).

Increases in chromosome number are more difficult to interpret because of the well-known problem of centromere increase. There is no evidence in the Hawaiian Telmatogeton for polyploidy or aneupolidy of large chromosomes as mechanisms for an increase in the number of centromeres. White (1973) illustrates a model for the dissociation of a metacentric chromosome into two acrocentric chromosomes. This model involves a reciprocal translocation between a large metacentric chromosome and a small chromosome. The new acrocentrics receive their euchromatic arms from the large metacentric chromosome and their additional telomeres and centromere from the small chromosome. The portion of metacentric chromosome without a centromere receives a centromere and a telomere from the small chromosome, and the portion of metacentric chromosome with a centromere receives only a telomere from the small chromosome. The key feature in increasing the chromosome number is that the small chromosome must not be a regular member of the karyotype. This additional chromosome may be an accessory chromosome or may have originated through nondisjunction. Only small chromosomes largely devoid of genetic material are ordinarily tolerated in nondisjunction.

A phylogeny which includes chromosome dissociations is necessary if T. abnormis is ancestral to the other freshwater forms. Metacentric chromosomes may have been transformed into acrocentric chromosomes by the mechanism discussed above. For each dissociation there may have been first a nondisjunction event of a dot chromosome providing the donor chromosome, a translocation between the dot and the metacentric chromosome producing two acrocentric chromosomes, and finally the fixation of the acrocentric chromosomes in a population. Three separate sets of dissociations would be required from T. abnormis (n = 4) to T. torrenticola-Maui (n = 7) and T. fluviatilis (n = 7).

The choice of a phylogenetic scheme depends on the selection of the primitive species. A scheme based on a species with a high chromosome number as being primitive appears in Fig. 16. Reduction in chromosome number according to this scheme occurs by centric fusion which is facilitated by the association of centric heterochromatin

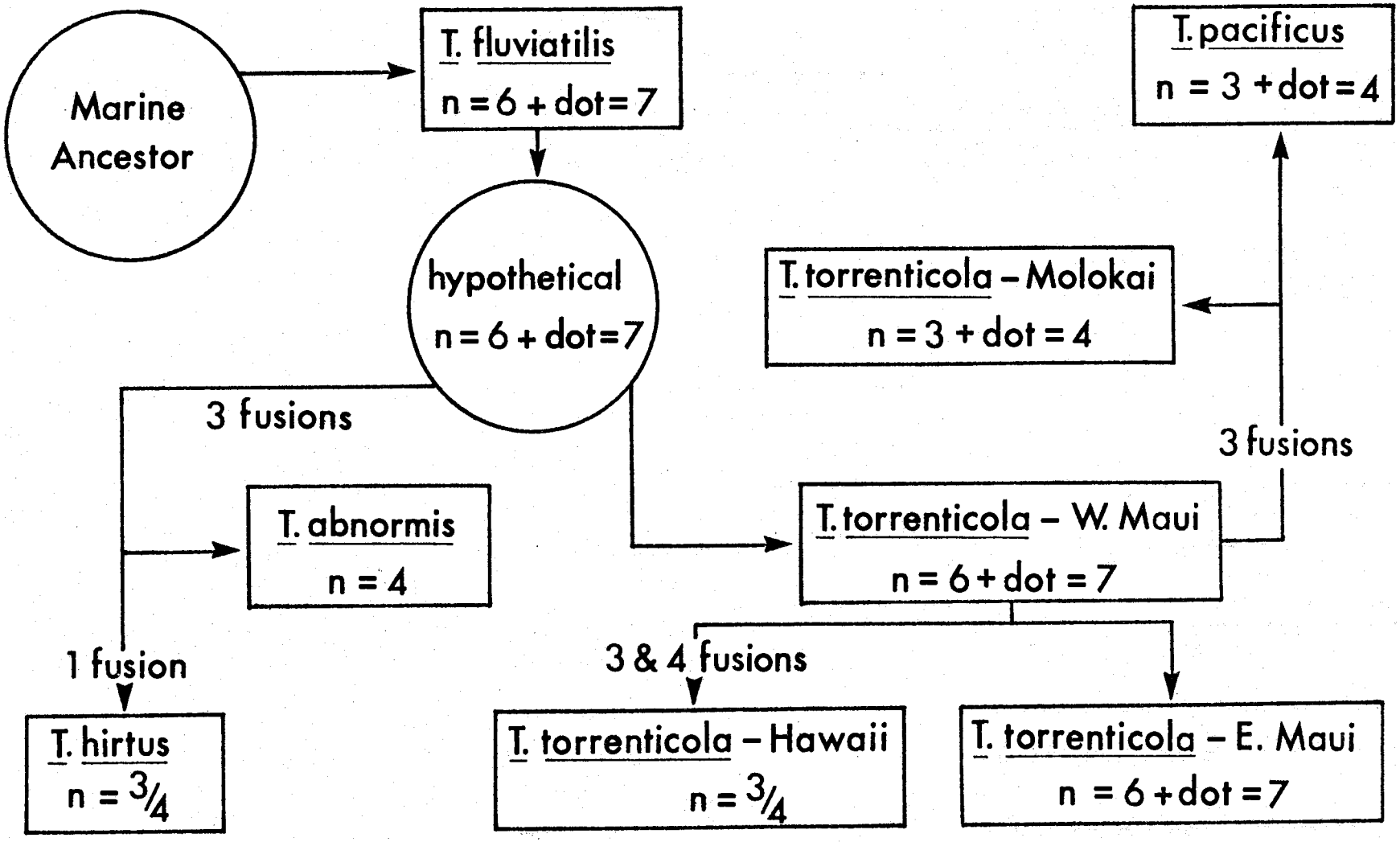


Fig. 16. Phylogenetic scheme for the Hawaiian Telmatogeton.

from all of the chromosomes in the chromocenter of interphase cells. Species without sex chromosomes are considered to be more primitive than those with sex chromosomes, and once attained, it is considered unlikely that such heteromorphic systems would be lost.

There are no data available which may help in deciding between T. torrenticola-W. Maui and T. fluviatilis as the primitive species. Interisland movements, however, are somewhat easier to comprehend if T. fluviatilis is taken to be primitive. Evolution of the Hawaiian Telmatogeton may thus have begun on Oahu with T. fluviatilis giving rise to a hypothetical species with one fixed inversion ($C_5 \rightarrow C^+$) and no change in chromosome number (Fig. 16). From the hypothetical species three fixed inversions and three sets of chromosome fusions could have given rise to T. abnormis-Oahu. A colonization could have established T. abnormis on Kauai which could have then led to the evolution of T. hirtus. T. abnormis and T. hirtus are thought to be related in spite of the lack of inversion data because they have the same fusion of polytene elements, both have element EF as the X-chromosome, and neither species has a dot chromosome. T. hirtus is thought to be distant from T. abnormis because its sex chromosome system is very complex and its polytene banding is very different.

The colonization of West Maui by the hypothetical species may have started a T. torrenticola-T. pacificus line of evolution with T. torrenticola-W. Maui being ancestral with seven pairs of chromosomes. Three fusions and three fixed inversions may have given rise to T. torrenticola-Molokai. T. pacificus and T. torrenticola-Molokai may be related since both have the same karyotype. T. torrenticola-Hawaii and T. torrenticola-E. Maui are thought to be rather closely related because they share inversion GH_4. The inversion data indicate that they emerged from T. torrenticola-W. Maui. Three and four fusions and two fixed inversions separate T. torrenticola-Hawaii from T. torrenticola-W. Maui. In the evolution of T. torrenticola-E. Maui, the chromosome number remained constant and there were at least five fixed inversions.

If T. abnormis is to be considered ancestral to the other freshwater species a complicated hypothesis is necessary. A hypothetical ancestor must be constructed since T. abnormis does not contain a dot chromosome and does have a differentiated sex chromosome. The hypothetical ancestor containing two pairs of metacentric chromosomes, two pairs of acrocentric chromosomes, and a pair of dot chromosomes ($n = 5$) could have given rise to T. abnormis with a fusion of the dot to an acrocentric chromosome and the differentiation of the sex chromosomes. Another fusion

could have produced the T. hirtus karyotype. The ancestor could have undergone two sets of dissociations to produce the ancestor of the T. torrenticola-T. fluviatilis species with 7 pairs of chromosomes. Chromosome fusions could then have led to the T. torrenticola-Molokai species with a lower chromosome number. This scheme is considered most unlikely since it involves a complicated array of dissociations.

In summary, the data of this study do not support the phylogeny proposed by Wirth (1947). Either T. torrenticola-W. Maui or T. fluviatilis is considered to be the ancestral freshwater species. There are two distinct lines of evolution--one with T. abnormis and T. hirtus and the other with the four T. torrenticola species. T. pacificus may represent a return to the marine environment. Centric fusions are believed to have been responsible for the chromosome number changes which structured the polytene elements into chromosomes and established a complex sex chromosome system in T. hirtus and T. torrenticola-Hawaii.

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